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1.0 Purpose

This procedure describes the steps to be followed in initiating and maintaining mammalian cell cultures from frozen stocks.

2.0 Scope

This SOP applies to Biopharmaceutical Development Program (BDP) personnel who are practicing GMP cell culture. It may also be used as a reference for other, non-GMP cell culture.

3.0 Authority and Responsibility

3.1 The Program and Technical Director, Technical Operations, BDP has the authority to define this procedure.

3.2 The Program and Technical Director, Technical Operations, BDP is responsible for ensuring adherence to this procedure.

3.3 Biopharmaceutical Quality Assurance (BQA) is responsible for area clearance prior to the initiation of cultures manufactured in accordance with Current Good Manufacturing Practices (cGMP).

3.4 BQA is responsible for quality oversight of this procedure.

4.0 Materials and Equipment

4.1 Pipets, BDP PN's: 20101, 20103, 20104, 20100, 20102, 20105, 20099.

4.2 Assorted Flasks, BDP PN's: 20074, 20076, 20077, 20745, 20531, or BDP approved equivalent.

4.3 Sterile collection container.

4.4 Pipetting device.

4.5 15 mL centrifuge tubes, BDP PN 20006, or BDP approved equivalent.

4.6 12 x 75 mm test tubes, BDP PN 20147, or BDP approved equivalent.

4.7 Decon ahol (70% IPA), BDP PN 30129.

4.8 0.4% Trypan Blue, BDP PN 10095.

4.9 Water Bath.

4.10 CO_2 Incubator.

4.11 2 to 8°C Refrigerator.

4.12 -10 to -30°C Freezer.

4.13 Hemacytometer, BDP PN 20739.

4.14 LUNA II automated cell counter

5.0 Procedure

Perform all steps aseptically in a certified Biological Safety Cabinet, per ***SOP 19102 - Routine Use and Disinfection of Biological Safety Cabinets, Incubators, Shakers, and Centrifuges.***

5.1 General Guidelines

These are general guidelines for established cell lines. Each cell line will have unique characteristics. Media formulations, split rations, and Trypsin formulations (adherent cells only) will need to be determined for each cell line.

5.1.1 Before beginning work, log into the Biological Safety Cabinet and disinfect per ***SOP 19102*** - ***Routine Use and Disinfection of Biological Safety Cabinets, Incubators, Shakers, and Centrifuges.***

5.1.2 Complete Medium Preparation - Disinfect all bottles and supplies (when possible) with the appropriate disinfectant and place inside the Biological Safety Cabinet. Compound the complete media desired. Use one pipette per addition.

5.1.3 Label the bottle(s) with the cell line name, date made, initials of preparer, lot number of product (if applicable), ingredients, and a 28-day expiration date on the bottle(s) of complete medium. Store complete media and all components at the recommended temperature and in the dark when not in use. Record in laboratory notebook, MPR, and/or **Form 13209-01** as applicable.

5.1.4 Turn on the water bath and warm to 37°C ± 2°C.

5.1.5 Pre-warm the complete medium to 37°C ± 2°C in a water bath or incubator before using.

5.2 Initiating Cells from Frozen Stocks

5.2.1 Prepare the work area in the Biological Safety Cabinet before thawing the cells. This includes disinfecting the pre-warmed media bottle and collecting the necessary supplies (flasks, pipettes, pipetter, centrifuge tubes, etc.).

5.2.2 Withdraw the cells from storage and transfer to the appropriate laboratory. Maintain the storage temperature until ready to thaw (a cup of dry ice is ideal).

5.2.3 Prepare a centrifuge tube with 9 mL of pre-warmed complete medium. The volume of media may be adjusted depending on the density of cells in the vial.

Thaw the vial(s) of cells in the water bath until just thawed. Do not let the vial seal get wet.

5.2.4 Quickly transfer the vial to the Biological Safety Cabinet and rinse the outside with Decon ahol. Remove the contents with a pipette and add to the centrifuge tube and medium. The vial may be rinsed with medium to collect all the cells.

5.2.5 A sample can be taken at this point or after centrifugation to count and check the viability of the cells (**Form 13209-02**, Cell Split Record Sheet may be used). Remove a sample of the cells and count. Refer to the following SOPs:

- **SOP 12227 - Operation and Maintenance for the LUNA II Automated Cell Counter**
- **SOP 13214 - Using a Hemocytometer to Determine Density, Viability, Generation Time and Doubling Time for Mammalian Cells**
- **SOP 13240 - Operation and Maintenance of the Leitz DMIL Microscope**

5.2.6 Centrifuge the cells at 1000 RPM for 10 minutes at 2 to 8□C*),* or what is optimal for the cells. When the centrifugation is complete, transfer them to the BSC and quickly decant off the supernate into a sterile container. Resuspend the cell pellet in the complete medium. Refer to Section 5.3.3 for initial seeding. Place the cell suspension in a labeled flask(s). Adherent cells do not generally need to be spun down post thaw.

5.2.7 Label the top or side of flask with the cell line name, date, and lot number (if applicable). Only loosen cap if using buffer and cap is not vented and lay bottom side down in the CO_2 incubator 5% CO_2, (or what is optimal for the medium used) at least 70% humidity). The bottom of the flask is the largest surface area of plastic that will hold the most liquid.

NOTE: If the medium contains a buffer i.e. HEPES, the flask cap does not need to be loosened.

5.2.8 Disinfect and log out of the Biosafety Cabinet per ***SOP 19102 - Routine Use and Disinfection of Biological Safety Cabinets, Incubators, Shakers, and Centrifuges.***

5.3 Suspension Cultures

Suspension cells do not generally adhere to a substrate under normal growing conditions. The protein concentration in the growth medium and/or positive charge of the plastic may cause some to stick. Some cells that are considered suspensions will stick to plastic, but can be dislodged without a cell scraper or the use of enzymes in most cases.

5.3.1 The cells are incubated and counted until it is determined the cells are ready to split based on density, good viability and experience working with the cells.

5.3.2 Turn on the water bath or incubator and allow it to reach 37°C ± 2°C. Prepare the work area in the Biological Safety Cabinet before working with the cells. This includes disinfecting the pre-warmed media bottle and collecting the necessary supplies (flasks, pipettes, pipetter, centrifuge tubes, etc.).

5.3.3 Determine the optimal seed density for the cells by trying several concentrations. To calculate the volume of cells needed for each split, follow the formula below.

Initial seed/mL X total volume in new flask wanted ÷ live cells/mL in the flask or RB to be split = volume of cells needed.

Aspirate the cells several times with a pipette to obtain a uniform mixture. Some suspension cells will stick to the flasks and can be removed by forcing media with a pipette against the plastic. Add the required volume of cells and fresh medium to a new flask(s) (suggested volumes: 25 cm^2 flask - working volume 10 mL, 75 cm^2 - 20 mL, 150 cm^2 - 100 mL, 850 cm^2 roller bottle - 400 mL) and proceed with Steps 5.2.7 and 5.2.8 above. The volume with each passage can be increased when more cells are needed (Example: cell banking, bioreactor inoculum, optimizing growth conditions, etc.).

NOTE: Some suspension cells will tend to stick on treated plastic. To help alleviate this problem, there are cell culture vessels made just for suspension cultures. Examples are BDP PN's 20076, 20077, and 20889.

5.3.4 Document in laboratory notebook, MPR, and/or on Form 13209-02 and/or 13209-04 as applicable.

5.4 Adherent Cells

Adherent cells normally stick to the surface of a growth substrate and must generally be dislodged by mechanical or chemical means. Growth is in the form of a monolayer or sheet of cells filling the surface area of the growth vessel. Passaging the cells is usually judged on the percent confluency and cells/cm^2 not cells per milliliter.

After the procedure has been performed in Section 5.2 and placed in the CO_2 incubator, the cells will settle and stick to the plastic surface. Between each passage of cells, a record is kept of the percent confluency.

5.4.1 Passing the Cells

- Turn on the water bath or incubator and allow it to reach 37°C ± 2°C. Warm the complete medium and Trypsin to 37°C ± 2°C. Prepare the work area in the Biological Safety Cabinet before working with the cells. This includes disinfecting the pre-warmed media bottle and collecting the necessary supplies (flasks, pipettes, pipetter, centrifuge tubes, etc.).
- Aspirate or decant the supernate from the flask into a sterile container.
- Add the selected Trypsin solution to the flask (suggested volumes: 25 cm^2 - 5 mL, 75 cm^2 - 10 mL, 150 cm^2 - 20 mL and 850 cm^2 roller bottle - 50 mL). Rinse the cell sheet with the Trypsin and decant all but enough to keep the cell sheet moist.

NOTE: The cell sheet may be pre-rinsed with either non-serum containing media, PBS, or Trypsin before adding the final Trypsin rinse if the cells are difficult to remove from the plastic. Ensure that all of the pre-rinse solution is removed before adding the Trypsin.

- Incubate the cells at 37°C for about 2-5 minutes or longer until they release from the plastic. In some cases, it may help to tap the side of the flask to dislodge the cells. Resuspend the cells in fresh medium.
- Aspirate the suspension until uniform and free of clumps. Count the cells.
- Transfer the required cell volume to a new flask(s). Adherent cell splits are either stated in ratios of flask surface area or cells/cm2. Example: at a 1:6 split ratio, a 25 cm2 confluent flask can make a 150 cm2 flask. To split adherent cells based on cells/cm2 (surface area), use the formulation below.

  Viable cells/cm^2 wanted in initial seed x Total cm^2 in new flask = Total viable cells needed

  Total Viable Cells Needed = Total Volume of cells needed from suspension

  Viable cells/mL in suspension

- Add fresh medium and gently mix cells (working volume - 25 cm2 flask - 10 mL, 75 cm2 - 20 mL, 150 cm2 - 100 mL 850 cm2 roller bottle - 200 mL). The volume of media in the flask for adherent cells is not critical. Label the top or side of the flask with the cell line name, date, and lot number (if applicable). Only loosen the cap if not using buffer and the cap is not vented. Lay the flask bottom side down in the CO2 incubator (5% CO2, at least 70% humidity)

  NOTE: If the medium contains a buffer, i.e., HEPES, the flask cap does not need to be loosened. Do not move adherent cells after splitting until the next day.

5.4.2 Record splits in laboratory notebook, MPR and/or Form 13209-03 and/or Form 13209-04 as applicable.

NOTE: Some adherent cell lines respond well to re-feeding between splits. Remove all media in the flask and add back the same volume of fresh complete growth medial. The frequency is cell-line dependent.

6.0 Documentation

6.1 Maintain records in authorized notebooks or Master Production Records. Each page is numbered, dated, and initialed in black ink (Refer to ***SOP 21409 - Good Documentation Practices and SOP 21408 - Laboratory Notebooks Control and Use.***

6.2 Maintain permanent record of cGMP-documented workscopes in authorized notebooks, approved Batch Production Records or other forms as required.

7.0 References and Related Documents

7.1 **SOP 12227** *Operation and Maintenance of the LUNA II Automated Cell Counter*

7.2 **SOP 13214** *Using a Hemocytometer to Determine Density, Viability, Generation Time and Doubling Time for Mammalian Cells*

7.3 **SOP 13240** *Operation and Maintenance of the Leitz DMIL Microscope*

7.4 **SOP 19101** *Eppendorf Pipette Operation and Cleaning*

7.5 **SOP 19102** *Routine Use and Disinfection of Biological Safety Cabinets, Incubators, Shakers, and Centrifuges*

7.6 **SOP 21408** *Laboratory Notebooks Control and Use*

7.7 **SOP 21409** *Good Documentation Practices*

7.8 **Form 13209-01** *Media Formulation Log*

7.9 **Form 13209-02** *Scale-Up Suspension Cells*

7.10 **Form 13209-03** *Scale-Up Adherent Cells*

7.11 **Form 13209-04** *Cell Split Record*

8.0 Change Summary